

Original papers

Polymorphisms of the Pfatpase 6 and Pfcrt gene and their relationship with the *in vitro* susceptibility to dihydroartemisinin and chloroquine of *Plasmodium falciparum* isolates from Abobo, Côte d'Ivoire

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ABSTRACT. As a result of widespread resistance to chloroquine (CQ) and sulphadoxine-pyrimethamine (SP), artemisinin-based combination therapy (ACT) has been recommended as a first-line anti-malarial regimen in Côte d'Ivoire since 2005. A thorough understanding of the molecular bases of *P. falciparum* resistance to existing drugs is therefore needed. The aims of this study were to analyze the *in vitro* sensitivity of *P. falciparum* field isolates from Abobo to CQ, pyronaridine (PYR) and dihydroartemisinine (DHA), and to investigate the polymorphisms associated with drug resistance. The standard *in vitro* drug sensitivity microtechnique recommended by the WHO was used to assess the sensitivity of *Plasmodium falciparum* isolates collected in December 2006. The Pfcrt haplotype 76 was analysed by PCR-RFLP while Pfatpase 6 amplification products were sequenced. Associations between drug sensitivity and parasite gene polymorphisms were evaluated with Cohen's kappa test. The correlation between the IC50 values for different drugs was assessed by the coefficient of determination (r^2). Significance was assumed at $p<0.05$. Of 128 *in vitro* tests performed, 112 (87.5%) were successful. Of the isolates, 56.2% were resistant for CQ and 48% for PYR. One isolate (3.6%) demonstrated reduced DHA sensitivity (IC50 higher than 10 nM). The mutant K76T pfcrt codon, present in 90% of DNA fragments analyzed, was associated with CQ-R ($\kappa=0.76$). The N669Y (16.1%), D734Y (28.6%) and D734H (1.8%) isolates were found to have mutant Pfatpase6, however, these mutations were not associated with diminished DHA sensitivity ($k=0.01$). These high levels of antimalarial drug resistance in Abobo (Côte d'Ivoire) demand further studies of drug efficacy across the whole country.

Key words: drug resistance, Pfatpase 6, pfcrt, Côte d'Ivoire

Introduction

Despite increased funding on malarial control programs, malaria remains a major public health problem, with approximately 781,000 deaths in 2009 [1]. Due to its geographical situation in sub-Saharan Africa, Côte d'Ivoire is facing many cases of malaria, especially among children under five years of age, in whom the prevalence of symptomatic and asymptomatic carriers has been found to be approximately 43% [2]. Many African countries have changed their treatment regimen for malaria after learning of chloroquine resistance in *Plasmodium falciparum*. In 2005, Côte d'Ivoire adopted Artemisinin-based combination therapy (ACT) for first-line treatment of uncomplicated malaria and sulphadoxine-pyrimethamine in preventive treatment for pregnant women. In Abidjan in 2002, chloroquine and sulphadoxine showed were found to have a treatment failure rate

of 34% among children aged 1 to 5 years [3].

Several molecular markers have been proposed to act as key contributors to antimalarial drug resistance in *P. falciparum*. The K76T mutation in the *Pfcrt* gene confers resistance to chloroquine [4]. This marker has been clinically correlated with resistance in Africa, at least in areas where the strain sensitivity is not negligible. *In vitro* tests have found the S108N mutation in the *Pfdhfr* gene to be associated with of parasite resistance to pyrimethamine, and the presence of other mutations at codons 51, 59 and 164 is known to increase the level of resistance [5]. Molecular monitoring is now included in the *P. falciparum* sensitivity surveillance strategy promoted by the WHO, in association with *in vivo* tests. Resistance to chloroquine has been linked to a *Pfcrt* gene mutation located on chromosome 7, [6] and clinical assays have confirmed this association [7–8].

The *dhfrS108N* mutation, carried by chromosome 4, is the key mutation site for resistance to pryrimethamine, and this is modulated by mutations of SNPs 51 and 59. The triple mutation *dhfrS108AN, dhfrC59R, dhfrN51I* has been shown to be selected in failures of the sulphadoxine-pyrimethamine association (SP) [9–10]. The *dhps* gene on chromosome 8 codes for dihydropteroate synthetase (*dhps*), and mutations of this gene are linked to sulphamide resistance. Mutations *dhpsS436A, dhpsA437G, dhpsK540E and dhpsA581G* have been described [11]. The *dhpsA437G*, *dhpsK540E* double mutation or the *dhpsS436A*, *dhpsA437G*, *dhpsK540E* triple mutation are known to be linked to higher resistance levels [10].

Apart from these genes, the sarco/endoplasmic reticulum Ca^{2+}-ATPase orthologue of *P. falciparum* (*Pfatpase6*) was suggested to be involved in the mechanism of action and resistance of the parasite to artemisinins. L263E, S769N, E431K and A623E single nucleotide polymorphisms (SNPs) in *Pfatpase6* have been proposed to be associated with reduced parasite sensitivity to artemisinins [12–13].

It should be noted that little research has been performed on *Pfatpase* 6 in Côte d'Ivoire. *In vivo*, *in vitro* and molecular analyses have been performed over the last few years to help prevent the development of resistance to each component drug and reduce the overall transmission of malaria [14]. The aim of this study was to determine the association between the presence of the gene mutation and amplification polymorphisms *pfatpase6* and *Pfcrt*, and the *in vitro* sensitivity of clinical *P. falciparum* isolates to CQ, PYR and DHA in Abobo (Abidjan), Côte d'Ivoire, an area with multidrug-resistant *P. falciparum.*

Materials and Methods

Drugs. Dihydroartemisinin (DHA), chloroquine (CQ) and pyronaridine (PYR) were obtained from Sigma Aldrich®. A stock solution of dihydroartemisinin was prepared in 70% methanol while stock solutions of chloroquine and pyronaridine were prepared in sterile distilled water. Two-fold serial dilutions of the stock solutions were prepared in RPMI 1640 medium. The final concentrations of the drugs tested ranged from 12.5 to 1,600 nM for chloroquine, 1.25 to 160 nM for pyronaridine and 0.5 to 64 nM for dihydroartemisinin.

Maturation of *P. falciparum* isolates. Clinical isolates of *P. falciparum* were obtained from patients before they were treated. Venous blood samples (5mL) were collected in EDTA-coated tubes (Terumo Europe N.V., Leuven, Belgium) from patients who gave their informed consent. Giemsa-stained thin and thick blood smears were examined to check for mono-infection with *P. falciparum* and to determine parasite density. Maturation assays were performed in 96-well tissue culture plates. Each well contained 200 µL of parasite suspension at 1.5% hematocrit and different drug concentrations. The plates were maintained for 42 h at 37°C in a candle jar as previously described [15]. Parasite growth was stopped by freezing at −20°C for at least 3 hours. Patients were treated by artemether-lumefantrine (AL) according to the recommendations of the national program for malaria control. The study was reviewed and approved by the Ivorian National Ethics Committee. All patients gave verbal consent before blood collection.

***In vitro* assay.** Venous blood samples were washed three times in RPMI 1640 medium. The erythrocytes were resuspended in complete RPMI 1640 medium (RPMI 1640 supplemented with 10% type O^+ human serum), 25 mM HEPES buffer, and 25 mM sodium bicarbonate) at a haematocrit of 1.5% and initial parasitaemia of 0.1–0.5%. If the parasitaemia > 0.5%, fresh uninfected erythrocytes were added to adjust it (0.1–0.5%). The sensitivity of isolates to antimalarial drugs was assessed using the World Health Organization (WHO) microtest technique, and the inhibition of schizont maturation

was measured microscopically. A suspension of infected erythrocytes (200 µL) was distributed in each well of the 96-well tissue culture plates containing the antimalarial drug solutions. After incubation, parasites were harvested and Giemsa-stained thick blood films were prepared. The number of mature schizonts (defined as parasites with > 3 nuclei) was counted per 200 asexual parasites. Isolates with less than 20% of mature schizonts in the control well were excluded.

Sample collection and DNA extraction. Blood samples were collected at the El Rapha and Anokoua Kouté health centers from patients with uncomplicated *P. falciparum* infection prior to drug treatment. All patients were from the Abobo health district in the period 2006-2007. The initial diagnosis was made by microscopic examination of Giemsa-stained thick blood films or a rapid diagnostic test. For the molecular test, approximately 200 µL of capillary blood from infected patients, with a haemoglobin rate > 6 g/dL, was taken by finger prick and imbibed onto filter paper (Isocode Stix®, Schleichler & Schuell, Ecquevilly, France). After the blood was taken, all patients were treated free of charge with AL (ACTs) recommended by the malaria control program. The imbibed blood filter paper samples were dried and stored for molecular analysis at University of Paris-Sud XI, UMR 8080, Orsay (France). DNA was extracted from the filter papers by the boiling method as follows: after rinsing two times with 500 µL of distilled water, the filter papers were immersed in 75µL of distilled water in a 0.5mL micro-tube and incubated at 99°C for 30 min. For each PCR, 10µL of the supernatant was used.

PCRs. After rinsing with 500 µl of distilled water, the filter papers were immersed in 75 µL of distilled water in a 0.5 mL micro-tube and incubated at 99°C for 30 min. For each PCR, 10µL of the supernatant was used. The following mixture was prepared in a final volume of 50 µL: Genomic DNA (10 µL supernatant), specific primers (10 pM), buffer (Tris 10 mM, pH 8.3, KCl 50 mM, $MgCl_2$ 1.5 mM), dNTP 0.2mM, and one unit of *Taq* DNA polymerase.

The primary PCR was performed with TCRP-1 (5'-CCG-TTA-ATA-ATAAAT-ACA-CGC-AG-3') and TCRP-2 (5'-CGG-ATG-TTA-CAA-AAC-TAT-AGT-TAC-C-3') for *Pfcrt*, and PFATP-1 (5'ATP-GAA-GAA-GAG-GTT-ATT-AAG-AAT-GCT-CAT-ACA) and PFATP-2R (5'ATT-CAT-GGT-TCA-TTT-TTA-TAT-GGT-TGT-TTA) for region A, PFATP-4 (5' GAT-TCT-TTA-ACA-GAA-TAC-CAA-CTA-TGT-CAA) and PFATP-5R (5' TGC-CAT-ATG-GCT-GGT-ATA-CGT-GTA-TTT-ATG) for region B for *Pfatpase* 6. The PTC-100 thermal cycler (MJ Research, Watertown, MA) was programmed to perform 30 cycles of 94°C for 2 min (first cycle) or 1 min (29 cycles), 50°C (56°C for *Pfcrt*) × 1 min, and 72°C × 1 min, followed by 72°C × 12 min at the end of 30 cycles.

The secondary PCR was performed with primer pairs TCRD-1 (5'-TGT-GCT-CATGTG-TTT-AAA-CTT-3') and TCRD-2 (5'-CAA-AAC-TAT-AGTTAC-CAA-TTT-TG-3') for *Pfcrt* (200 bp fragment) and PFATP-3 (5'GGT-TTG-AAT-GAA-TTA-GAA-GTA-GAA-AAG-AAG) for region A and (5' ACA-GAA-TAC-CAA-CTA-TGT-CAA-AAA-GGG-GAT) for region B for *Pfatpase 6*. The *Pfcrt* PCR products were digested by *Apo* I (PCR-RFLP) (50°C,Tris-HCl 50mM, $MgCl_2$ 10mM, NaCl 100mM, DDT 1mM), while the *Pfatpase 6* amplification products were sequenced. Electropherograms were analyzed using Edit view sequence analysis software (PerkinElmers, Les Ulis, France). The wild-type *Pfcrt* was defined by the allele K76.

Test analysis. IC_{50} values with 95% confidence intervals (CI) were calculated by using an Emax model (available at http://www.antimalarial-icestimator.net) as RE=100 – [(100*Cγ)/(Cγ + IC50γ)], where IC_{50} is the Drug Inhibiting Concentration at 50% of parasite activity, γ is a sigmoidicity factor which expresses the steepness of the curve, RE is the relative effect of the drug (as a percentage, Y-axis), and C is the drug concentration (X-axis). The IC_{50} cut off values for resistance to chloroquine and pyronaridine were 100 nM and 15 nM, respectively, and the cut-off for diminished susceptibility to DHA was 10 nM. The associations between drug sensitivity and parasite gene polymorphisms were evaluated with Cohen's kappa test [16]. The degree of agreement was scored as follows: 0–0.20, slight agreement; 0.21–0.4, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, good agreement; and > 0.81, very good agreement.

Results

In vitro drug sensitivity of *P. falciparum* isolates

A total of 45 *P. falciparum* isolates were

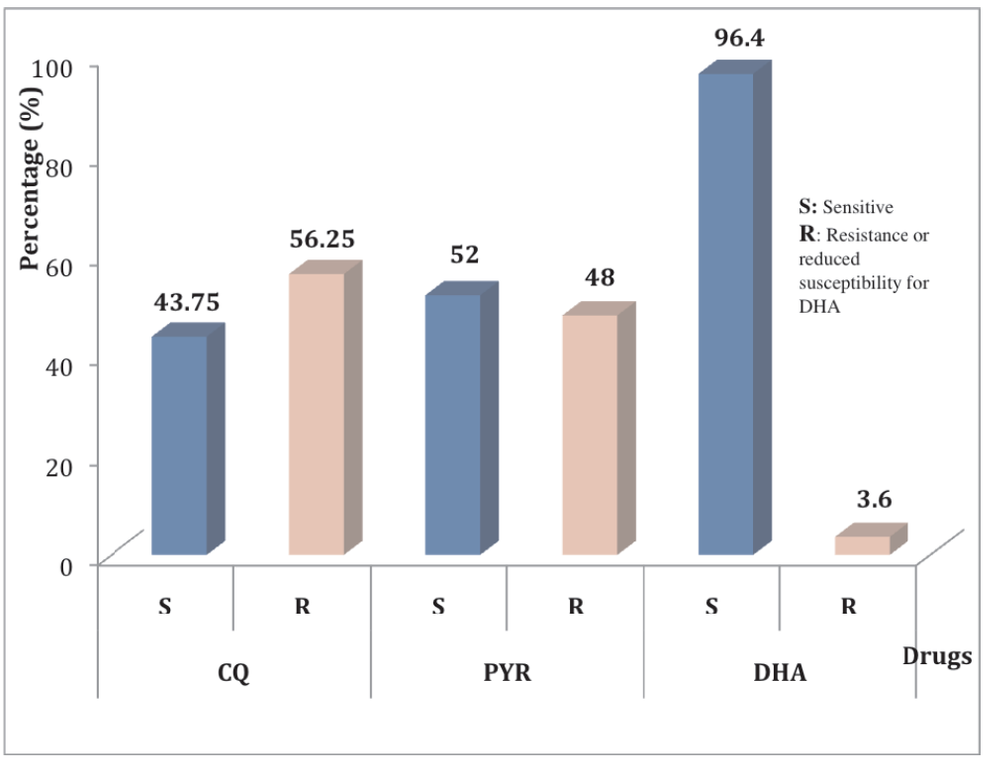

Fig. 1. *In vitro* susceptibility of *Plasmodium falciparum* isolates to chloroquine (CQ), and pyronaridine (PYR), and dihydroartemisinin (DHA)

collected from the Anonkoua Kouté and El Rapha health centers. Only 32 (78%) had a parasite density > 0.1% and a total of 128 *in vitro* tests have been performed. A total of 112 assays (87.5%) were successful, and Table 1 shows the corresponding geometric mean IC_{50} ($GMIC_{50}$) values determined from these assays for each drug tested.

Dihydroartemisinin (DHA) was the most active drug tested against *P. falciparum* isolates: Only 3.6% (1/28) of the parasites had reduced susceptibility to this drug against 97.4% (27/28) of sensitivity *P. falciparum* isolates. The following proportions of resistance observed for the other drugs were 56.25% and 48% for chloroquine and pyronaridine respectively (Fig. 1). The geometric mean CQ IC_{50} value was 145.5 (65–226) nM, and 56.2% (n=32) of isolates were CQ-resistant. The geometric mean PYR IC_{50} value was 17.7 (9.1–26.3) nM, and 48% (n=27) of isolates were PYR-resistant. The geometric mean DHA IC_{50} value was 2.7 (1.45–3.99) nM, and 3.6% (n=28) of isolates had reduced DHA susceptibility. Eleven isolates were sensitive to all three drugs. A moderate level of cross-resistance was observed. As can be seen in Table 1**,** a positive correlation was found between the activities of DHA and PYR (r^2=0.40, p<0.05), as well as between the activities of CQ and PYR (r^2=0.45, p<0.05). DHA activity did not correlate with CQ activity (r^2=0.29, p=0.08) (Table 1).

Drug sensitivity and *P. falciparum* gene polymorphism

PCR-RFLP analysis (enzymatic digestion) of DNA fragments from the *P. falciparum* isolates (n=18) indicated that 55.6% were K76T mutants (n=10) and 44.4% were wild-type K76 (wt K76) (n=8). Of 18 chloroquine resistant (CQ-R) isolates, 88.9% were *Pfcrt* mutant T76 (n=16) against 11.1% wild K76 (n=2).

Table 1. Correlation of antimalarial drugs activities

Drug 1	Drug 2	Coefficient of determination (r^2)	P
CQ	PYR	0.45	<0.05
DHA	CQ	0.29	0.08
PYR	DHA	0.40	<0.05

Table 2. Distribution of *pfcrt* polymorphism according to drugs sensitivities

Antimalarial drugs profile	Number (%) of isolates with *pfcrt* K76T		κ*
	K	T	
CQ Resistant ($IC_{50} \geq 100$ nM)	2 (11.1%)	16 (88.9%)	0.76
CQ Sensitive ($IC_{50} \leq 100$ nM)	12 (85.7%)	2 (14.3%)	
PYR Resistant ($IC_{50} \geq 15$ nM)	9 (69.2%)	4 (30.8%)	0.21
PYR Sensitive ($IC_{50} \leq 15$ nM)	8 (57.1%)	6 (42.9%)	
DHA Reduced susceptibility ($IC_{50} \geq 10$ nM)	1 (100%)	0 (0%)	< 0.0
DHA Sensitive ($IC_{50} \leq 10$ nM)	8 (29.6%)	19 (70.4%)	

* Kappa test of Cohen: The degree of agreement was scored as follows: 0-0.20, slight agreement; 0.21-0.4, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, good agreement; and > 0.81, very good agreement

On the other hand, of 14 chloroquine-sensitive isolates, two were *Pfcrt* mutant K76T (14.3%) and 12 were wt K76 (85.7%), kappa coefficient k=0.76. This reflects a good agreement between the mutation at position 76 *Pfcrt* and sensitivity of *P. falciparum* to chloroquine.

Thirteen isolates were found to be resistant to pyronaridine (PYR-R) of which 4 *Pfcrt* were mutant K76T (30.8%) and 9 were wt K76 (69.2%), while 14 were susceptible isolates (PYR-S), comprising 6 mutant K76T (42.9%) and 8 wt K76 (57.1%). There is a slight correlation between the sensitivity of *P. falciparum* to PYR and *Pfcrt* mutation at position 76 (k=0.21).

Of 28 isolates tested against DHA. only one isolate (wt K76) was found to have reduced

Table 3. Distribution of *Patpase 6* polymorphisms according to antimalarial drugs sensitivities

Antimalarial drugs profile		Polymorphisms *of Pfatpase 6* (number of isolates)						κ*
		Haplotype 669		Haplotype D 734Y		Haplotype D734H		
		N**	**Y**	D	**Y**	D	**H**	
CQ[1]	Resistant ($IC_{50} \geq 100$ nM)	17 (94.4%)	1 (5.6%)	13 (72.2%)	5 (27.8%)	18 (100%)	0 (0.0%)	0.25
	Sensitive ($IC_{50} \leq 100$ nM)	13 (92.8%)	1 (7.2%)	9 (64.3%)	5 (35.7%)	13 (92.8%)	1 (7.2%)	
PYR	Resistant ($IC_{50} \geq 15$ nM)	12 (92.3%)	1 (7.7%)	6 (46.1%)	7 (53.9%)	12 (92.3%)	1 (7.7%)	0.16
	Sensitive ($IC_{50} \leq 15$ nM)	13 (92.8%)	1 (7.2%)	5 (35.7%)	9 (64.3%)	14 (100%)	0 (0.0%)	
DHA	Reduced susceptibility ($IC_{50} \geq 10$ nM)	1 (100%)	0 (0.0%)	1 (100%)	0 (0.0%)	0 (0.0%)	1 (100%)	0.01
	Sensitive ($IC_{50} \leq 10$ nM)	19 (70.4%)	8 (29.6%)	11 (40.7%)	16(59.3%)	27 (100%)	0 (0.0%)	

[1] CQ: chloroquine; PYR: pyronaridine; DHA: dihydroartemisinin

*Kappa test of Cohen: The degree of agreement was scored as follows: 0-0.20, slight agreement; 21-0.4, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, good agreement; and > 0.81, very good agreement

** N: Asparagine; Y: Tyrosine; D: Aspartic acid; H: Histidine

sensitivity. Of the 27 isolates sensitive to DHA, 8 isolates were wt K76 (29.6%) and 19 were mutant K76T (70.4%) (Table 2).

Regarding the *Pfatpase* 6 gene polymorphism, N669Y (16.1%) is relatively high as 734 haplotypes (30.4%) with D734Y (28.6%) and D734H (1.8%). The codons of the polymorphism and their involvement with malaria are given in more detail in Table 3.

Discussion

The high level of chloroquine resistance observed since 2003 has prompted the Malaria Control Program to develop their strategy for reducing malaria prevalence. Drug resistance arises rarely but spreads relatively quickly. The most spectacular activity of chloroquine is its ability to increase from nanomolar levels outside the parasite to millimolar levels in the digestive vacuole of the erythrocyte trophozoite [17]. All CQ-R isolates demonstrated an alteration in the accumulation of chloroquine in the food vacuole, which is thought to be due to an alteration of the pH gradients and /or membrane permeability as a result of an efflux mechanism. It now appears that CQ resistance is associated with a decreased uptake of the molecule. Drug accumulation has been observed to be highly structurally specific, which means either a carrier / specific permease is involved, or a molecule associated with hematin in the food vacuole [18].

The results of the present study confirm that the K76T mutation plays a significant role in the occurrence of *Pfcrt* CQ-R (κ=0.76) as reported by Djimde et al. (2001) [7]. A similar study conducted in Côte d'Ivoire in 2007 showed a relationship between the presence of the *Pfcrt* K76T mutation and therapeutic failure of chloroquine [4]. Moreover, the present findings indicate that mutations *Pfcrt* K76T/*Pfatpase* N669Y or *Pfatpase* D734H could exert a synergistic effect. All isolates of *P. falciparum Pfcr* / *Pfatase* double mutants are CQ-R.

All isolates of *P. falciparum* apart from the twofold N669Y and D734H mutant are sensitive to DHA. In accordance with information for isolates from Cambodia, French Guiana, and Senegal, none of the isolates observed in our study carried single nucleotide polymorphisms of *pfatpase6* at codon 769 or at codons 37, 693 or 898 [13]. These results are consistent with those of Zhang et al. (2008) from isolates of Chinese origin which note that codon 769 is not associated with reduced sensitivity of *P. falciparum* to artemisinin derivatives [19].

However, an important genetic polymorphism in the *Pfatpase6* gene has 23 point mutations throughout the entire gene but has no direct impact on the susceptibility of isolates to CQ or DHA. The kappa coefficient obtained is not significant. This lack of correlation, despite the large number of point mutations, may sometimes exist because they did not rise to a characteristic location of the gene. Within codon 734, the substitutions D734Y (28.6%) and D734H (1.8%) were observed. This could significantly alter the codons involved in the changes of molecular target [20] which are enzyme ATPase-calcium dependent.

Although the mechanism of action of artemisinins remains unclear, one proposal is based on its interaction with the sarcoplasmic reticulum Ca^{2+} ATPase 6 (*Pfatpase 6*). This enzyme, when expressed in *Xenopus* oocytes, was specifically inhibited by artemisinin derivatives containing an endoperoxide bridge [21]. In addition, the activity of the enzyme was greatly influenced by the introduction of several gene mutations. An analysis of naturally occurring polymorphisms in *Pfatpase 6* in field isolates from French Guyana suggests that a polymorphism at codon 769 may be associated with reduced susceptibility of these isolates to artemether *in vitro* [12]. However, other studies have failed to detect any codon 769 polymorphism in field isolates [10,22]. The results of our study do not indicate any link between either amplification or mutation of *Pfatpase6* and the *in vitro* response to DHA. Both 669 and 734 are haplotypes identified in this study, and considering the frequency of mutation and physico-chemical changes of the amino acids, it can be assumed that there are two new allelic sites in the *Pfatp6* gene. This gene has been identified as the genetic basis of the reduction of resistance of *P. falciparum* to artemisinin derivatives, as the presence of new point mutations in *Pfatp6* genes has been associated with reduced effectiveness of artemisinin derivatives [23,24].

Conclusions

These data showed that the resistance of *P. falciparum* is indeed a reality in Cote d' Ivoire. Till 2003, Chloroquine was the first-line therapy, despite its removal, continues to have a high level of resistance. It was shown in this study that CQ-R is bonded to mutation of codon *Pfcrt* K76T, but no

mutation linked with artemisinin resistance in *Pfatpase* 6. Despite the decrease in efficiency, DHA is the best alternative for the treatment of uncomplicated malaria. In addition, molecular epidemiology should be part of routine surveillance to produce complementary information to assess the appropriateness of the current national anti-malarial drug policy.

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